

some instances, may be co-localized. More recently, quantum dots are widely used in immunofluorescence staining for the biomarkers of interest due to their intense and stable fluorescence.

**[0010]** Identifying the individual constituent stains for the biomarkers and the proportions they appear in the mixture is a fundamental challenge that is solved using a spectral unmixing operation. Spectral unmixing decomposes each pixel of the multi-spectral image into a collection of constituent spectrum end members or components, and the fractions of their intensity contributions in the multi-spectral image from each of them. An example spectral unmixing method is a non-negative linear least squares operation commonly used both in fluorescent and brightfield microscopy. WO 2015/101507 (PCT/EP2014/078392) and WO 2015/124772 (PCT/EP2015/053745) which are incorporated herein by reference in their entirety disclose various unmixing methods for unmixing a multichannel image (also referred to as multispectral image).

#### BRIEF SUMMARY OF THE INVENTION

**[0011]** Provided herein are methods of detecting and describing heterogeneity in a cell sample comprising at least one analyte labelled with a detectable marker, said methods comprising analyzing an image of the cell sample on a computer apparatus comprising a computer processor programmed to apply a cluster analysis to a dataset obtained from the image of the cell sample to create a cluster map comprising a plurality of clusters of expression patterns, wherein:

**[0012]** (a) the dataset comprises an image stack for each of a plurality of fields of view (FOV) within one or more areas of interest (AOI) of the cell sample, wherein the image stack comprises a x-axis, a y-axis, and a z-axis, wherein the x-axis and the y-axis represent spatial coordinates within the field; and the z-axis comprises one or more layers, wherein each layer of the z axis comprises intensity data for a single detectable marker at a plurality of x,y coordinates; and

**[0013]** (b) the cluster analysis comprises applying an unsupervised, non-parametric, density-based clustering algorithm to the image stacks, wherein the clustering algorithm groups x,y coordinates with other x,y coordinates having a similar ratio of detectable marker intensity across layers of the z-axis, thereby generating the plurality of clusters having similar expression patterns.

**[0014]** A 'cell sample' as understood herein is any biological tissue sample, such as a surgical specimen that is obtained from a human or animal body for anatomic pathology. The cell sample may be a prostate tissue sample, a breast tissue sample, a colon tissue sample or a tissue sample obtained from another organ or body region.

**[0015]** A 'multi-spectral' or 'multi-channel' pixel as understood herein encompasses a pixel contained in a digital image obtained from a biological cell sample in which different nuclei and tissue structures are simultaneously stained with specific dyes.

**[0016]** A 'multi-channel image' or 'multi-spectral' image as understood herein encompasses an image that is composed of multi-spectral or multi-channel pixels. A single channel image is obtained for each of the channels of the multi-channel image by means of an unmixing method.

**[0017]** In an embodiment, the density based clustering algorithm is a Mean-Shift clustering algorithm. In another embodiment, the dataset of the foregoing methods is obtained by a method comprising:

**[0018]** (a1) calculating a FOV sampling grid (which optionally comprises a plurality of FOVs at regularly spaced intervals across the AOI) for each of a plurality of AOI within the image;

**[0019]** (a2) automatically collecting multi-spectral data and/or hyper-spectral data at single or multiple z-planes in each FOV (which optionally may be automatically saved in a nested data structure or data base with metadata attributes, said metadata attributes comprising patient, assay, biopsy, section, AOI position, and/or FOV position);

**[0020]** (a3) computationally segmenting detectable marker signals from the multi-spectral data and/or hyper-spectral data;

**[0021]** (a4) selecting FOVs to be compared as a group in the cluster analysis into a dataset structure, wherein, optionally,

**[0022]** (a4a) the FOVs selected to be compared as a group correspond to different tumor foci in the same tissue section or

**[0023]** (a4b) the FOV are grouped on the basis of a biopsy taken from the same patient for comparison to a different biopsy taken from the same patient; or

**[0024]** (a4c) FOVs are grouped on the basis of tumor location; or

**[0025]** (a4d) FOVs are grouped based on the patient for comparison to another patient; or

**[0026]** (a4e) FOVs are grouped on the basis of tumor genotype; and

**[0027]** (a5) applying automatic morphological feature segmentation to each detectable marker signal of each FOV in the data set, said feature segmentation optionally being based on size constraints, intensity constraints, or a combination of size constraints and intensity constraints.

**[0028]** In another embodiment, the method for obtaining said dataset of the foregoing methods further comprises:

**[0029]** (a6) manually designating regions in one or more FOVs to include or exclude from the cluster analysis.

**[0030]** In another embodiment, the detectable marker of the foregoing methods generates a signal that is separable from other markers and tissue on basis of spectral or other physical characteristics when co-localized, and quantifiable. In an embodiment, the detectable marker is attached to an antibody or an antigen-binding fragment thereof. In an exemplary embodiment, the detectable label is attached to at least one antibody that specifically binds to at least one phosphorylated protein (such as, for example, a member of PI-3 kinase signal transduction pathway or MAP kinase signal transduction pathway). In a further embodiment, the cell sample that is labeled with the anti-phospho antibody is a tissue that was fixed using a two-temperature fixation.

**[0031]** Also provided herein are methods of characterizing a tumor according to physiological state of a signal transduction pathway in the tumor, the methods comprising analyzing an image of a sample of the tumor according to the foregoing methods, wherein:

**[0032]** two or more analytes are labeled with the detectable marker;